Published in final edited form as:

Clin Transl Oncol. 2019 March ; 21(3): 342–347. doi:10.1007/s12094-018-1930-4.

Measurement of circulating tumor cells in squamous cell carcinoma of the head and neck and patient outcomes

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Abstract

Objectives—We report the outcomes of patients with squamous cell carcinoma of the head and neck (HNSCC) whose circulating tumor cells (CTCs) were quantified using surface-enhanced Raman scattering (SERS) nanotechnology.

Methods—SERS tagged with EGF was used to directly measure targeted CTCs. Patient charts were retrospectively reviewed. An optimal cut point for CTCs in 7.5 ml of peripheral blood predictive of distant metastasis-free survival (DMFS) was identified by maximizing the log-rank statistic. An ROC analysis was also performed.

Results—Of 82 patients, 13 experienced metastatic progression. The optimal cut point for DMFS was 675 CTCs ($p = 0.047$). For those with distant recurrence ($n = 13$) versus those without distant recurrence ($n = 69$), the CTC cut point which results in the largest combined sensitivity and specificity values is also 675 (sensitivity = 69%, specificity = 68%).

Conclusion—Liquid biopsy techniques in HNSCC show promise as a means of identifying patients at greater risk of disease progression.

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Compliance with ethical standards

Conflicts of interest The authors declare they have no conflicts of interest.

Research involving human participants and/or animals This study was approved by our institutional review board and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

This research was presented in abstract form at the annual meeting of the American Radium Society; May 6–9, 2017; Colorado Springs, Colorado.

Keywords

Neoplastic cells, circulating; Precision medicine; Carcinoma, squamous cell of head and neck; Prognosis; Neoplasm metastasis

Introduction

Cancers of the upper aerodigestive tract are the sixth most common malignancy in the world, with squamous cell carcinoma accounting for 95% of these cases [1, 2]. Despite aggressive therapy, 5-year survival for all stages combined is roughly 50%. Fifty to sixty percent of patients will fail locoregionally, while 20% will ultimately develop distant metastases [3]. The presence of cervical lymph node metastases is a major negative prognostic factor for head and neck squamous cell carcinoma (HNSCC) [4, 5].

Of increasing interest is HPV positive disease. A majority of patients presenting with squamous cell carcinoma of the oropharynx in particular have HPV positive disease, which has been found to be a strong prognostic factor for survival [6]. With this has come interest in treatment de-escalation for these patients. However, even patients with HPV positive disease have similar rates of distant metastases to their HPV negative cohorts [6]. There is a need for sensitive and specific methods of prediction for patients at high risk of locoregional and distant recurrence. Non-invasive means of cancer detection via peripheral blood samples, termed “liquid biopsies,” are one potential method of determining metastatic potential in HNSCC.

If localregional control is excellent, metastatic disease represents the greatest threat to survival. Circulating tumor cells (CTCs) as a condition for metastases was first hypothesized by Thomas Ashworth in 1869 [7]. CTCs are shed from primary tumors into the circulatory system and can lead to the formation of clinical metastases [8]. CTCs have been associated with metastatic disease and decreased survival [3, 9–11]. CTCs may serve as a tool for personalized medicine. Sensitive and specific assays are required for detection of CTCs in peripheral blood given the rarity of these cells in circulation compared to blood cells, and their ambiguous biology [12, 13]. Over the past decade, advances in the detection of CTCs, along with other liquid biopsy techniques, have been made [9, 12, 14–17]. Our group previously developed a direct assay based on highly specific and sensitive surface-enhanced Raman scattering (SERS) nanotechnology to detect CTCs in peripheral blood, which requires no subsequent separation procedure [16]. The SERS nanoparticles used in our method are able to amplify the Raman scattering efficiencies of adsorbed molecules by as much as 10^{14} - to 10^{15} -fold, allowing spectroscopic detection and identification of a single molecule under ambient conditions (Fig. 1) [18]. In this study, we report the outcomes of patients with squamous cell carcinoma of the head and neck whose circulating tumor cells were quantified using this novel technique.

Materials and methods

After institutional review board approval, peripheral blood samples from 125 patients with stage I–IVC or locoregionally recurrent squamous cell carcinoma of the head and neck

treated at Winship Cancer Institute at Emory University, Atlanta, were collected. Collection occurred between December 2009 and August 2011. The method for detection of CTCs has been previously described [16]. SERS nanoparticles tagged with EGF was used to directly measure targeted CTCs in the presence of white blood cells. In brief, the SERS nanoparticle was conjugated with EGF peptide. Lower density CTCs and leukocytes were isolated from whole blood by density-gradient centrifugation. They were then incubated with EGF-SERS particles. Free excess nanoparticles were removed by three cycles of gentle centrifugation. The cell pellet was illuminated by a 785 nm laser, and a SERS spectrum was taken to record the level of targeted nanoparticles. SERS signal intensity corresponded to the number of CTCs in the presence of WBCs. Due to technical errors, the circulating tumor cells in 25 patients could not be quantified. Patients who did not receive treatment at our institution or who did not return for follow-up were also excluded from the study, leaving 82 patients for analysis. The charts of these patients were retrospectively reviewed.

Statistical analysis

Distant metastasis-free survival (DMFS) was defined as time from date of sample collection to date of progression. Those without distant recurrence were censored at date of last follow-up. An optimal cut point for CTCs in 7.5 ml of peripheral blood was identified by maximizing the log-rank statistic [19].

A separate analysis was run to identify a cut point for CTCs in 7.5 ml peripheral blood between those with no distant recurrence versus those with a distant recurrence. An ROC analysis was performed, and area under the curve (AUC), sensitivity, and specificity were reported. CTCs in 7.5 ml peripheral blood were compared across event type using ANOVA, and descriptive statistics were also reported. The statistical analysis was performed using SAS 9.4, and the statistical significance was assessed at the 0.05 level.

Results

Patient and tumor characteristics

The median follow-up time from CTC collection was 51.9 months (range 0.33–85.5 months). The male:female distribution was 60:22, and the median age at collection of CTCs was 58.5 years (range 23–77 years). The median number of circulating tumor cells per 7.5 ml blood sample was 401 cells (range 0–6908 cells/7.5 ml). The largest percentage of patients had primary squamous cell carcinoma of the oropharynx ($n = 33$), and 34.1% had stage IVA disease at time of collection. At the time of CTC collection, 47 patients were being treated definitively for a new diagnosis of localized disease and 10 were treated for locally recurrent disease. Twenty patients were in remission after undergoing definitive treatment for localized disease. In addition, five had metastatic disease when CTCs were collected. Most patients were treated with concurrent chemoradiation ($n = 52$). Patient and tumor characteristics are delineated in Table 1.

Outcomes

Thirteen patients experienced metastatic progression. Of these patients, eight had CTCs measured prior to development of metastatic disease. Nine of the 13 patients had CTCs >

500 (range 0–6908 CTCs/7.5 ml). One patient with metastatic disease was found to have a biopsy proven isolated metastasis at T9. CTC count at that time was 88 cells/8 ml. He received 24 Gy in one fraction to T9 followed by four cycles of Taxol, Carboplatin, and Ifosfamide. His subsequent CTC count was undetectable, but he eventually expired due to further metastatic disease. Ten patients had or developed local disease progression from the time of peripheral blood collection, with only four of said patients having CTCs > 500.

CTC statistics by event type are included in Table 2. The optimal cut point for DMFS was 675 CTCs, which resulted in a log-rank p value of 0.047 (Fig. 2). Five-year DMFS was 89% for those with CTCs ≤ 675 and 71.4% for those with CTCs > 675. In the analysis of no distant recurrence ($n = 69$) versus those with distant recurrence ($n = 13$), the area under the curve was 0.661 (Fig. 3). The CTC cut point which results in the largest combined sensitivity and specificity values is 675. At 675, the sensitivity is 69%, and the specificity is 68%.

Discussion

In our study, a direct assay utilizing SERS technology was used to detect CTCs in peripheral blood, which has the advantage of not requiring a separation procedure given the sharp spectral pattern associated with SERS. EGFR was utilized given its known overexpression in HNSCC. Thirteen patients experienced metastatic progression. Nine of those 13 patients had CTCs > 500, and the optimal cut point for DMFS was 675 CTCs ($p = 0.047$). Interestingly, one patient with metastatic disease had detectable CTCs at the time of discovery of an isolated bone metastasis which became undetectable after radiation treatment, yet he still died of distant disease. For those with no distant recurrence versus those with distant recurrence, the CTC cut point which results in the largest combined sensitivity and specificity values was 675. This resulted in a sensitivity of 69% and a specificity of 68%.

Multiple techniques have been developed for the detection of CTCs in peripheral blood samples. The most common detection methods are PCR-based molecular assays and immunological assays in which monoclonal antibodies are directed against histogenic proteins [20]. A majority of these techniques require enrichment of the CTCs prior to detection. CellSearch™ has been approved by the FDA for detection of CTCs in peripheral blood in patients with metastatic breast, prostate, and colorectal cancers [21, 22]. In this technique, CTCs are enriched via magnetic beads conjugated to antibodies against EpCAM and labeled with a fluorescent dye. The tumor cells are identified by cytokeratin staining with said antibodies. The sample is counterstained with fluorescently labeled antibodies conjugated with CD45 for hematopoietic cells. Identification of CTCs is then carried out with a fluorescence-based microscopy system [9, 20]. This system has shown promising results in the detection of CTCs in breast, prostate, and colorectal cancers. In a prospective, multicenter study utilizing CellSearch™, Cristofanilli et al. tested the peripheral blood samples of 177 patients with metastatic breast cancer for CTCs prior to initiation of a new line of treatment. They found patients with CTCs ≥ 5 per 7.5 ml of whole blood had shorter median progression-free survival and overall survival compared with the group with fewer than five circulating tumor cells per 7.5 ml [9]. In our study, we found 672 CTCs per 8 ml of peripheral blood to be predictive of the development of metastatic disease. This is perhaps

due to the high sensitivity of SERS. Also using CellSearch™, Cohen et al. found the baseline CTC count is prognostic in patients with metastatic colorectal cancer [21]. Further, Moreno and colleagues showed CTCs could be quantified in patients with metastatic prostate cancer and serve as a correlate of disease progression [23].

Precision medicine is a rapidly changing field and, more recently, molecular analysis of CTCs and additional types of liquid biopsies have been developed. Molecular analysis of CTCs allows for the monitoring of changes in the tumor genome and can have important implications [24]. For instance, determination of new mutations in patients with non-small cell lung cancer (NSCLC) can assist in selection of therapy and provide prognostic information [25]. More recently, circulating tumor DNA (ctDNA) has been explored as a means of liquid biopsy. ctDNA is largely found in plasma and is thought to enter the circulation via primary and metastatic tumor deposits and lysis of CTCs [26]. Multiple methods, including PCR technologies, have been used to detect known tumor-specific tumor mutations and genome-wide alterations [26, 27]. Like CTCs, ctDNA has been found to be a valid method for measuring tumor burden and treatment response for multiple tumor types. Studies have evaluated whether numbers of CTCs and ctDNA are similar in individual patients with varying results showing ctDNA is likely not derived entirely from CTCs [25, 28–30]. However, ctDNA has also been found to be a possible surrogate for extent of disease [26, 27, 30, 31]. Chaudhuri et al. quantified molecular residual disease using Cancer Personalized Profiling by Deep Sequencing ctDNA analysis after treatment of localized lung cancer. In 72% of patients, ctDNA detection preceded radiographic progression by a median of 5.2 months. In addition, 52% of patients had mutations that predict for response to tyrosine kinase inhibitors or immune checkpoint inhibitors. This adds to the idea of personalized medicine as intervention when disease burden is low and may allow for improved outcomes.

In the head and neck realm, methods of liquid biopsy show great promise as a means of personalized treatment for those with HPV + SCC of the oropharynx. Recent protocols, including NRG HN002 and ECOG-ACRIN 3311, are de-escalating both radiation and chemotherapy for patients without high-risk features. However, systemic therapy will be needed for some percentage of these patients given the known risk of distant metastases in this population [6]. Thus, for patients with HPV + disease and a high level of CTCs at diagnosis, de-escalation of systemic therapy may not be appropriate. The results of our study show measurement of CTCs can be used as a means of predicting for development of distant metastases, potentially leading to changes in upfront therapy for those with high levels of CTCs in the peripheral blood.

Limitations of our study include its retrospective nature and small sample size. In addition, CTCs were collected at a single time-point for all but one patient, and CTCs were collected at varying stages of disease and treatment. Also, while EGFR has been found to be overexpressed in 80–90% of HNSCCs, cells without this overexpression would have been missed [32]. However, the surface-enhanced Raman scattering (SERS) nanotechnology used for detection of CTCs in our study is sensitive and specific. Further, no separation procedure is required adding to the efficiency of the technique. Circulating tumor cells show great promise in the personalization of treatment for patients with HNSCC. Further studies are

needed to assess the link between CTCs/ctDNA and clinically relevant disease. Implications of liquid biopsy analysis in treatment are related to its use as a surrogate for metastatic potential and treatment response. Sensitive, specific, and timely assays could potentially be used for intensification or de-intensification of upfront treatment and alteration of treatment for poor response.

In conclusion, we used a novel technique for detection of CTCs in the peripheral blood of patients with HNSCC, which had a high detection rate. The optimal cutoff point for development of distant metastases was 675 CTCs per 7.5 ml peripheral blood. Five-year DMFS was 89% for CTCs ≤ 675 versus 71.4% for CTCs > 672. Further studies are underway to confirm the utility of liquid biopsy techniques as a means of identifying patients at greater risk of disease progression, potentially allowing for the personalization of upfront therapy.

Acknowledgments

Funding Research reported in this publication was supported in part by the Biostatistics and Bioinformatics Shared Resource of Winship Cancer Institute of Emory University and NIH/NCI under award number P30CA138292. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Fig. 1.
Representative SERS spectrum for patient one vs. control

CTCs in 7.5ml of blood sample	No. of Subject	Event	Censored	Median Survival (95% CI)	1 Yr Survival	2 Yr Survival	5 Yr Survival
≤675	52	5 (10%)	47 (90%)	NA (NA, NA)	91.3% (78.6%, 96.7%)	89.0% (75.6%, 95.3%)	89.0% (75.6%, 95.3%)
>675	30	8 (27%)	22 (73%)	NA (NA, NA)	79.3% (59.5%, 90.2%)	71.4% (50.6%, 84.6%)	71.4% (50.6%, 84.6%)

Fig. 2.
Distant metastasis-free survival—CTC

Fig. 3.
Analysis of local/no ($n = 69$) vs. distant ($n = 13$) recurrence

Table 1

Patient and treatment Characteristics

Variable	Level	N = 82	%
Primary site	Oropharynx	33	40.2
	Sinonasal	3	3.7
	Larynx	17	20.7
	Oral cavity	18	22
	Hypopharynx	4	4.9
	Nasopharynx	5	6.1
	Unknown primary	2	2.4
Sex	Female	22	26.8
	Male	60	73.2
Stage at time of CTC collection	I	2	2.4
	II	4	4.9
	III	3	3.7
	IVa	28	34.1
	IVb	10	12.2
	IVc	5	6.1
	Recurrent disease	10	12.2
	Remission	20	24.4
Tobacco	Yes	55	67.1
	No	27	32.9
Pack years	10	36	43.9
	> 10	46	56.1
Treatment	Concurrent chemoradiation	52	63.4
	Radiation	6	7.3
	Surgery	1	1.2
	Other combined modality	20	
	Palliative	3	6.3
Event type	No distant disease	69	84.2
	Distant disease	13	15.9
CTCs in 7.5 ml of peripheral blood sample	Mean	941	–
	Median	401	–
	Minimum	0	–
	Maximum	6908	–
	Std dev	1356	–
	Missing	0	–

Table 2

CTC statistics by event type

Covariate	Event type			
	Statistics level	No distant recurrence <i>N</i> = 69	Distant disease <i>N</i> = 13	<i>p</i> value*
CTCs in 7.5 ml of blood sample	<i>N</i>	69	13	0.017
	Mean	787.28	1755	
	Median	352.5	1027.5	
	Min	0	0	
	Max	5355	6907.5	
	Std dev	1111.67	2132.71	

Bold value indicates significant difference in CTC statistics by event type

* The *p* value is calculated by ANOVA